

A report of laboratory studies on the toxins of blue-green algae is presented. At least three types of toxin are known to be involved in algal poisonings. More work must be done to decide if there are other toxins, and what species of animals are susceptible to different toxins.

LABORATORY STUDIES ON THE TOXINS PRODUCED BY WATERBLOOMS OF BLUE-GREEN ALGAE

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THE STUDY of toxic waterblooms of blue-green algae was initiated in our laboratory about seven years ago to find out which of the common species of planktonic blue-green algae were toxic and whether or not they actually cause the sporadic outbreaks of poisoning reported from Australia, Canada, Israel, United States, Union of South Africa, USSR, and elsewhere.

Part of the material presented here has been published already^{3,6,8} but part represents recent unpublished findings.

When work began seven years ago we first examined the records of previous investigations (for reviews, see references 5-7, 9, 11, 15, 21) and concluded that the following four points about toxic waterblooms were more or less well established:

1. The occurrence of toxic waterblooms was sporadic and symptoms and survival times of poisoned animals varied considerably. Species composition of blooms varies greatly and this was thought to be the cause of differences in toxicity.

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2. At least six species of algae were suspected of toxicity. No species was definitely proved toxic but there were strong indications that two species in particular, *Microcystis aeruginosa* and *Anabaena flos-aquae*, were toxic.
3. Bacterial infections were not involved.
4. Recognized toxins, including botulinus toxin, were not involved. *Microcystis* toxin was thought to be an alkaloid.¹⁰

We also concluded that dependence upon the unpredictable occurrence and persistence of toxic waterblooms had handicapped previous investigations and probably had contributed to a number of conflicting results and conclusions that had been reported.

We decided, therefore, to adopt the laboratory culture approach, even though this necessitated spending large amounts of time and effort in learning how to isolate and to culture different species and strains of planktonic blue-green algae (many for the first time) on both a small and a moderately large scale. The more detailed aims of the investigation were to provide answers to the following four questions:

1. Why do blooms vary in toxicity?
2. Is more than one toxin involved and what are the origins and symptoms produced by each?
3. What is the chemical nature of the toxin or toxins?
4. How susceptible are different animals to the toxin or toxins?

The first species that we succeeded in isolating and growing uniaxially in the laboratory was a strain of *Microcystis aeruginosa*, termed NRC-1, that came from a nontoxic bloom. It proved to be toxic to white mice by both oral and intraperitoneal routes. We discovered that this alga, with its associated bacteria, produced two distinguishable toxic factors. One we called the fast-death factor (FDF) since, with mice, it caused pallor followed by violent convulsions, prostration, and death within about one hour after the administration of a minimal lethal dose either orally or intraperitoneally. The other one we called the slow-death factor (SDF) since, with mice, it caused piloerection, dyspnea, lethargy, and death within 4 to 48 hours after administration of a minimal lethal dose. It was generally less effective orally than intraperitoneally. The presence of FDF tended to obscure the presence of SDF, but the two factors could be distinguished because it was found that FDF was an endotoxin. Fresh suspensions of *Microcystis* NRC-1 cells caused either no deaths or slow deaths, while the same suspensions would cause fast deaths after they had been frozen and thawed, sonic disintegrated, or decomposed by semi-anaerobic incubation. This response was also confirmed with toxic blooms containing predominantly *M. aeruginosa*.

Extensive, but unsuccessful efforts were made to purify *M. aeruginosa* NRC-1 by means of antibiotics, disinfectants, bacteriostatic agents, ultraviolet irradiation, infinite dilution, and single-cell isolation. Since we were unable to purify the alga, we had to resort to an indirect proof that the alga was the source of FDF and the bacteria were the sources of SDF:

1. Several strains of *Microcystis* were isolated from different blooms. It was found that strains producing FDF, FDF plus SDF, or SDF could be isolated from the same bloom.

2. Strain NRC-1 was mass-cultured and algae-rich and bacteria-rich fractions were separated by differential centrifugation and tested for toxicity. The algae fraction produced FDF while the bacterial fraction produced SDF.
3. Five strains of bacteria were isolated from strain NRC-1 and some were found to produce SDF but none produced FDF.
4. Bacteria from strain NRC-1 failed to induce any production of FDF when added to a nontoxic strain of *Microcystis*.
5. One of two clones derived from single-cell isolates of strain NRC-1 produced FDF and the other produced SDF.

The effects of age, light intensity, temperature, and aeration upon the production of FDF by strain NRC-1 were examined. FDF production and accumulation were found to be correlated with good growth. FDF content declined rather rapidly when growth ceased. Only traces of FDF appeared in the medium of old cultures, indicating an apparently high rate of decomposition. The optimum temperature for production of FDF was found to be as much as 7° C lower than the optimum temperature for growth. We concluded, therefore, that the production of FDF by *Microcystis* was both genetically and physiologically controlled and that this explained the variable toxicity of *Microcystis* blooms. We also concluded that more than one toxin was frequently involved, and that slow-death factors produced by associated bacteria were the probable cause of some symptoms such as intestinal inflammation reported in connection with algal poisonings.

We next turned our attention to the problem of isolating and identifying FDF from *M. aeruginosa* NRC-1. Large quantities of algal cells were required and this necessitated expanding the scale of culture until we finally had a "factory" producing algal bloom in the laboratory at the rate of 1 to 2 kg of freeze-dried cells per month. This also enabled us, in collaboration with the Animal Diseases Research Institute of the Canada Department of Agriculture,

Table 1—Toxicity of Lyophilized Cells from 24 Waterblooms of Blue-Green Algae; Intraperitoneal, White Mice, Dosage Range 40 to 640 mg per kg Body Weight

Dominant Species	Blooms Producing:		
	Very Fast Deaths (1-10 Min)	Fast Deaths (1-2 Hr)	No Deaths or Slow Deaths (4-48 Hr)
<i>Anabaena flos-aquae</i>	5	—	3
<i>Aphanizomenon flos-aquae</i>	—	—	1
<i>Lyngbya birgei</i>	—	—	1
<i>Microcystis aeruginosa</i>	—	7	3
<i>Aphanizomenon</i> plus <i>Microcystis</i>	—	4	—
Total	5	11	8

to conduct tests on other animals besides mice. Suitable doses of lyophilized cells of strain NRC-1 proved toxic when administered orally or intraperitoneally to mice, guinea pigs, rabbits, chickens, sheep, and calves. Survival times were longer with the larger animals but all showed a characteristic type of liver damage. Domestic ducks were resistant to massive doses of *Microcystis* cells. This was interesting since it eliminates *Microcystis* FDF as a likely cause of waterfowl sickness. The symptoms and autopsy findings produced in susceptible animals agreed quite well with those reported by Wheeler, Lackey, and Schott,²² Ashworth and Mason,¹ or Shelubsky¹⁷ for poisonings by *Microcystis* blooms in the United States and Israel. Professor Douw G. Steyn in South Africa kindly tested lyophilized cells of strain NRC-1 on laboratory animals and reported that the effects were indistinguishable from those observed previously with *Microcystis* toxica during his investigation of algal poisonings in the Transvaal.¹⁸ We concluded, therefore, that *Microcystis* FDF is one of the important algal toxins.

Isolating and identifying the structure of *Microcystis* FDF proved difficult since

it was biologically active at concentrations below those that would give any measurable chemical reactions of a diagnostic nature. Extraction and purification had to be guided by bio-assay with mice. Finally, enough toxin was concentrated to establish that it was not an alkaloid but a polypeptide. However, it was contaminated with other polypeptides with similar properties. After many difficulties, these were resolved by electrophoresis on paper using borate buffer under mildly alkaline conditions. FDF proved to be one of a mixture of five closely related polypeptides. It has a low molecular weight and is probably cyclic. Hydrolysates contain seven amino acids in the following proportions:

aspartic	1
glutamic	2
D-serine	1
valine	1
ornithine	1
alanine	2
leucine	2
	—
	10

The LD₅₀ (IP) for mice of the purified toxin is only 0.47 mg/kg. Its structure resembles that of such other biologically active peptides as the antibiotics

bacitracin and gramicidins. *Microcystis* FDF showed no antibiotic activity when tested with the usual spectrum of bacteria, however.

While work with *Microcystis* proceeded, we continued to look for toxic blooms of other species from which to make isolates. We were particularly interested in locating toxic blooms of *Anabaena flos-aquae* or *Anabaena lemmermannii*, since reports by Fitch, et al.,⁵ Rose,¹⁴ Firkins,⁴ and Olson^{11,12} suggested that these produced a faster acting toxin which killed various animals and waterfowl and had different chemical properties from those of *Microcystis* FDF. To date we have tested the toxicity of 24 blooms from different sources (intraperitoneal, mice) (Table 1). To simplify tabulation, the numbers of blooms producing slow deaths have been combined with those producing no deaths. Minimal lethal doses of five blooms of *Anabaena flos-aquae* from Saskatchewan, collected during the summers of 1960 and 1961, caused very fast deaths preceded by mild convul-

sions, gasping, and paralysis. These blooms were toxic by the oral as well as the intraperitoneal route. Samples of one of these toxic blooms were sent to Dr. L. D. Jones in South Dakota and Professor Theodore Olson in Minnesota for comparative tests. Dr. Jones reported that it produced the same effects on mice as those observed with blooms of this species during the outbreak of poisonings at Storm Lake, Iowa, in 1952.^{4,14} Professor Olson indicated that the effects produced on mice were indistinguishable from those which he has observed with toxic blooms of *Anabaena lemmermannii*.^{11,12}

The seven toxic blooms of *Microcystis aeruginosa* (Table 1) all produced FDF. Four blooms consisting of *Aphanizomenon flos-aquae* plus *Microcystis aeruginosa* in approximately equal proportions were obtained from Klamath Lake, Ore. One was a sample collected in 1957 by Professor H. K. Phinney and dried at 30° C.¹³ With the assistance of Mr. C. A. Peek, the other three blooms were collected in the summer of 1960

Table 2—Toxicity of Lyophilized Cells and Culture Filtrates of 52 Strains of Blue-Green Algae; Intraperitoneal, White Mice, Dosage Range 40 to 640 mg per kg Body Weight

Species	Strains Producing:		
	Very Fast Deaths (1-10 Min)	Fast Deaths (1-2 Hr)	No Deaths or Slow Deaths (4-48 Hr)
<i>Anabaena flos-aquae</i>	5	—	2
<i>Anabaena limnetica</i>	—	—	3
<i>Anabaena spiroides</i>	—	—	2
<i>Anabaena scheremetievi</i>	—	—	2
<i>Anacystis cyanea</i> f. minor	—	—	9
<i>Anacystis nidulans</i>	—	—	2
<i>Aphanizomenon flos-aquae</i>	—	—	5
<i>Coelosphaerium kuetzingianum</i>	—	—	1
<i>Gloeotrichia echinulata</i>	—	—	1
<i>Microcystis aeruginosa</i>	—	8	11
<i>Nodularia sphaerocarpa</i>	—	—	1
Total	5	8	39

and lyophilized. As judged by the symptoms produced, all four blooms contained only *Microcystis* FDF.

From various blooms and other sources we have succeeded in growing a total of 52 strains representing 11 species of planktonic blue-green algae. The toxicity of each of these strains has been tested (intraperitoneal, mice) and the results classified according to the type of deaths produced (Table 2). Very fast deaths preceded by the same symptoms observed with the parent toxic blooms were produced by five out of seven strains of *Anabaena flos-aquae*. Eight out of 19 strains of *Microcystis aeruginosa* produced FDF. All other strains, including those of the suspect species *Aphanizomenon flos-aquae*, *Coelosphaerium kuetzingianum*, and *Gloeotrichia echinulata*, were either nontoxic or produced slow deaths.

The fact that toxic and nontoxic strains of *Anabaena flos-aquae* have been isolated indicates that the origin of very fast death factor (VFDF) is probably algal and that production is genetically controlled. With four of the five toxic *Anabaena flos-aquae* strains, the bulk of the toxin was located in the culture filtrates. We suspect that this was caused by leakage and lysis as the cultures aged. Since *Anabaena* VFDF acts more rapidly than *Microcystis* FDF it probably permeates cell membranes more rapidly and hence it probably has a lower molecular weight. The chemical structure of *Anabaena* VFDF and its effect upon other animals besides the mouse need further investigation. Preliminary tests indicate, however, that *Anabaena* VFDF and *Microcystis* FDF have similar solubilities in different solvents.

In summary, therefore, our investigation to date allows us to provide at least partial answers to the four questions asked at the beginning:

1. Blooms vary in toxicity because certain strains of certain species of algae are ca-

pable of producing toxins. These strains vary in dominance as well as in toxin production in response to environmental conditions. The production and release of bacterial toxins may modify or confuse the symptoms produced by the algal toxins.

2. At least three types of toxin are now known to be involved in algal poisonings. These can be distinguished by the symptoms they produce. *Microcystis* FDF is algal in origin and *Anabaena* VFDF appears to be algal in origin. There appear to be several slow death factors that are bacterial in origin.^{6,19,20}
3. *Microcystis* FDF has been identified as a probably cyclic polypeptide of moderately low molecular weight. The structures of *Anabaena* VFDF and of the bacterial toxins have not been established as yet.
4. *Microcystis* is more or less equally toxic to a variety of animals with the exception of the domestic duck. Since the potency of *Microcystis* FDF is low, rather large quantities of algae must be consumed to cause death. The degree of thirst or starvation of an animal will affect the amount of bloom consumed and, indirectly, contribute to variability in observed toxicity. We cannot make any firm statements about the susceptibility of different animals to *Anabaena* VFDF until more work has been done. However, there is much evidence from studies of blooms to suggest that it kills many species, including waterfowl.

Are there other toxins produced by other species of algae? Are recent reports of fish kills² and possible human poisonings¹⁶ caused by algae correct? These and other such questions about waterblooms will only be answered satisfactorily after further laboratory studies have been carried out.

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School of Public Health Building to Be Dedicated

Formal dedication of the University of North Carolina School of Public Health building will take place April 6 and 7, 1963, it has been announced by Edward G. McGayran, M.D., dean. Theme of the dedication program will be "Schools of Public Health: Past, Present, and Future." Included will be a series of symposia dealing with the role of schools of public health in contemporary society.

Alumni and public health practitioners in all fields of activity are invited to be present. Further information may be obtained by writing to the Dean, School of Public Health, Drawer 229, University of North Carolina, Chapel Hill, N. C.